

## A possible mechanism for the antiviral activity of pokeweed antiviral protein

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(Accepted for publication March 1993)

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The mechanism by which pokeweed antiviral protein (PAP) inhibits the infection of tobacco by TMV was investigated. The N-glycosidase activity of PAP was measured using a combination of aniline treatment to cleave the rRNA at the depurinated site, gel electrophoresis and Northern blot hybridization using a probe specific for the 3' end of tobacco 25S rRNA. Using this assay it was found that the host ribosomes, which are used by viruses to uncoat and to synthesize replicases at an early stage of infection, were depurinated and probably inactivated by PAP as early as 5 min after inoculation. It was also found that the extent of the inhibition of virus infection and the depurination of host ribosomes were positively correlated with the concentration of PAP. These results suggest that the depurination and inactivation of host ribosomes is the cause of the inhibition of virus infection by PAP.

### INTRODUCTION

Pokeweed antiviral protein (PAP) is one of a large group of plant proteins called ribosome-inactivating proteins (RIPs) [2]. Three forms of PAP were found in *Phytolacca americana* (pokeweed) leaves and seeds. They all consist of a single polypeptide with a molecular weight of 29–30 kDa [3, 15, 16]. PAP was first discovered in 1925 and since then, it and other RIPs have been tested extensively for their antiviral activities. Most of them have been shown to inhibit virus infection [6, 25]. Among them PAP has probably most potential as a virus inhibitor, as it can effectively inhibit infections by both plant and animal viruses from different groups [1, 5, 9, 10, 11, 17, 27–29]. Moreover, the barley translation inhibitor, a RIP from barley seeds, has been shown to inhibit the growth of fungi *in vitro* and in transgenic tobacco plants expressing the barley RIP [19, 23]. However, little is known about the mechanisms by which RIPs inhibit virus infection and the growth of fungi.

We are interested in the mechanism by which PAP inhibits the infection of tobacco by tobacco mosaic virus (TMV). PAP does not inactivate virus particles because virus infectivity is completely restored when virus and PAP mixtures are separated by

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Abbreviations used in text: PAP, pokeweed antiviral protein; RIP, ribosome inactivating proteins; TMV, tobacco mosaic virus.

ultracentrifugation or gel filtration [11, 27]. The inhibitory activity of PAP must therefore be achieved in an indirect way such as blocking certain events in host metabolism which are essential for virus replication. This simple mode of action seemed likely because it was demonstrated that PAP does not inactivate pokeweed ribosomes [21] and also it is less effective against virus infection of pokeweed than of other plants [12, 24]. However, this hypothesis is challenged by the recent finding that pokeweed ribosomes can be inactivated by endogenous PAP during their isolation [22, 26].

RIPs remove a specific adenine base in a conserved loop of the large rRNA of eukaryotic organisms [7, 8] and this modification (depurination) prevents the ribosomes binding to elongation factors, thereby blocking translation. This RIP-specific depurinated large rRNA is susceptible to amine-catalysed hydrolysis of the sugar-phosphate backbone at the depurination site which releases a small fragment of 230–400 nucleotides from the 3' end of the rRNA. This fragment is diagnostic for RIP-catalysed depurination and is readily observed following agarose/formamide gel electrophoresis [20]. This new method, based on the N-glycosidase activity of RIPs, has helped our investigation into the mode of action of PAP against virus infection. By this method, and using a PCR-amplified rRNA-specific probe for the diagnostic fragment, the mechanism of antiviral activity of PAP was shown to be associated with its ribosome inactivating ability.

## MATERIALS AND METHODS

PAP was purified from *Phytolacca americana* leaves as described previously [5]. Tobacco mosaic virus (TMV) was purified and stored at 4 °C. *Nicotiana tabacum* cv. Xanthi-nc plants were maintained in a glasshouse and used for bioassay.

### *Isolation of tobacco ribosomes and rRNA*

Tobacco leaves were frozen in liquid nitrogen and ground in 25 mM MgCl<sub>2</sub>, 25 mM KCl, 100 mM Tris-HCl, pH 8, in a mortar and pestle. The homogenate was filtered through cheesecloth and centrifuged at 25 000 *g* for 20 min. The supernatant was centrifuged again at 200 000 *g* through a cushion of 25 mM MgCl<sub>2</sub>, 25 mM KCl, 25 mM Tris-HCl, 1 M sucrose, pH 7.5 for 6 h. The crude ribosomes were resuspended in 25 mM MgCl<sub>2</sub>, 25 mM KCl, 25 mM Tris-HCl, pH 7.5, at 20 mg ml<sup>-1</sup> and stored at -80 °C.

rRNA was prepared from ribosomes by extracting with phenol/chloroform/iso-amylalcohol (25:24:1).

### *Preparation of total RNA*

Total RNA was isolated from tobacco leaves by a proteinase K lysis/LiCl precipitation procedure as described previously [18]. The isolated RNA was analysed by agarose gel electrophoresis to ensure that it was not degraded.

### *Depurination assay*

The assay was carried out as described by Hartley *et al.* [14]. Reaction mixtures containing 30 µg of tobacco ribosomes and various amounts of PAP in 25 mM MgCl<sub>2</sub>, 25 mM KCl, 25 mM Tris/HCl, pH 7.5, were incubated for 1 h at 30 °C. Control

reactions lacking PAP were incubated similarly. RNA was extracted using phenol/chloroform and dissolved in sterile distilled water at  $3 \mu\text{g ml}^{-1}$ . Aniline treatment of the rRNA and electrophoresis in agarose/formamide gels was done as described by May *et al.* [20].

#### *Generation of rRNA-specific probe*

The sequence 5'AGUACGA\*GAGGAACC 3' is highly conserved in 25S and 28S rRNA and is a universal site of action for PAP and other RIPs [8, 26]. The removal of A\* by PAP results in the release of an RNA fragment of about 350 nucleotides following aniline treatment. A probe specific to this 350-nucleotide fragment could be used to monitor sensitively and specifically PAP for its N-glycosidase activity.

The GenBank and EMBL sequence databases were searched for plant 25S rDNA sequences but no sequence data for tobacco rRNA was found. The 25S rDNA sequences from six plant species (citrus, cucumber, mung bean, rice, tomato and wheat) which were in databases were compared and two sequences [(i) 5' GTACGAGAGGAACCGTTG 3' within the PAP site of action, and (ii) 5' GCCACGATCCACTGAGAT 3' near the 3' terminal of 25S rDNA] were identified, which were identical in the 25S rDNA sequences of all six plants. It was hoped that these highly conserved sequences would also exist in tobacco 25S rDNA and two oligonucleotides designed from these sequences were synthesized for use as the 5' and 3' PCR primers.

The first strand cDNA was synthesized from total tobacco RNA using AMV reverse transcriptase (RT) using the oligonucleotide which was complementary to the 3' end of the RNA as a primer. The PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM  $\text{MgCl}_2$ , 0.01 % gelatin, 0.2 mM dNTPs, 0.1 % Triton X-100, 0.2  $\mu\text{g}$  of each primer, 1  $\mu\text{l}$  of 10-fold diluted RT reaction mixtures, 5 U Taq DNA polymerase in a total volume of 100  $\mu\text{l}$ . Each cycle of the PCR consisted of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s and primer extension at 72 °C for 45 s. The reaction was continued for 30 cycles. Reaction products were separated by electrophoresis on 1.5 % agarose gels and visualized by ethidium bromide staining. The PCR fragment was extracted once with chloroform and labelled with  $^{32}\text{P}$  using the nick-translation method.

#### *PAP antiviral activity*

*Time course.* Tobacco (*Nicotiana tabacum* cv. Xanthi-nc) plants were dusted with carborundum and mechanically inoculated with solutions containing PAP at  $2 \mu\text{g ml}^{-1}$  mixed with equal volumes of  $1 \mu\text{g}$  purified TMV  $\text{ml}^{-1}$ . Tobacco leaves inoculated with  $0.5 \mu\text{g}$  TMV  $\text{ml}^{-1}$  were used as controls. The inoculated leaves were washed thoroughly with distilled water immediately after inoculation. Two leaves were collected at 5 min, 10 min, 1 h, 5 h, 10 h and 15 h respectively after inoculation. Ten 6-mm diameter leaf discs were cut from each leaf (equivalent to about 120 mg) and were immediately frozen in liquid nitrogen in a pre-chilled mortar and ground to a fine powder using a pre-chilled pestle. The frozen powder was transferred to an Eppendorf tube and thawed in 300  $\mu\text{l}$  of RNA extraction buffer (0.2 M  $\text{H}_3\text{BO}_3$ , 30 mM EGTA, 1 % SDS) and 300  $\mu\text{l}$  of phenol/chloroform. The tube was vortexed for 1 min and the phases

separated by centrifugation at 14000 g for 5 min in a microcentrifuge. The aqueous phase was collected and again extracted with phenol/chloroform. The DNA was removed by digestion with DNaseI (Sigma) and the RNA purified by extraction with phenol/chloroform, precipitated with ethanol and finally dissolved in TE buffer. The RNA was treated with aniline, then fractionated by electrophoresis, transferred to a Hybond-N membrane and hybridized with the PCR-amplified rRNA-specific probe.

*Dose-response curve.* Tobacco (*Nicotiana tabacum* cv. Xanthi-nc) plants were mechanically inoculated with solutions containing PAP at 8 and 0.8  $\mu\text{g ml}^{-1}$ , and 80 and 8  $\text{ng ml}^{-1}$  mixed with equal volumes of 1  $\mu\text{g}$  purified TMV  $\text{ml}^{-1}$ . Tobacco leaves inoculated with 0.5  $\mu\text{g}$  TMV  $\text{ml}^{-1}$  were used as controls. The inoculated leaves were washed thoroughly with distilled water immediately after inoculation. Two leaves inoculated with PAP at each concentration were collected 1 h after inoculation. Ribosomes from each leaf were isolated, rRNA extracted and equal amounts were separated by denaturing agarose gel electrophoresis, transferred onto a membrane and hybridized with the probe. All assays were done at least twice with two replicates of each sample unless otherwise stated.

## RESULTS

### *Sensitivity of tobacco ribosomes to PAP*

The sensitivity of ribosomes from tobacco to PAP was investigated by detecting a diagnostic fragment released from 25S rRNA that had been treated with various amounts of PAP. Figure 1 shows an ethidium bromide-stained gel of RNA extracted from tobacco ribosomes incubated with PAP at different concentrations. Aniline treatment of rRNA from tobacco ribosomes incubated with PAP at concentrations from 0.1  $\mu\text{g ml}^{-1}$  to 100  $\mu\text{g ml}^{-1}$  caused the release of a fragment of about 350 nucleotides derived from the 3' region of the tobacco 25S rRNA at all PAP concentrations. Ribosomes from different plant species vary markedly in their sensitivity to PAP [4]. It is difficult to estimate the proportion of 25S rRNA that had been depurinated by PAP due to the presence of other bands on the gel. These bands are probably due to nicked rRNAs and/or chloroplast rRNAs which can account for up to 50% of the total population of ribosomes in the leaf cells [13] with cytosol intact rRNAs.

### *Specificity of the probe*

A probe specific to the diagnostic rRNA fragment was required for a more sensitive assay of the depurination of rRNA by PAP *in vivo*. Using primers based on the computer analysis, a 350-bp fragment was obtained by RT-PCR of tobacco rRNA. This was the expected size based on the positions of the primers on the rDNA sequences from six plant species and the size of the fragment released from tobacco rRNA after PAP treatment. To test the specificity of the probe, tobacco ribosomes were incubated with PAP at 1  $\mu\text{g ml}^{-1}$  and the corresponding rRNA treated with aniline as described above. Ribosomes not incubated with PAP or rRNA not treated with aniline were used as controls. As expected, a fragment of about 350 nucleotides was released only from aniline-treated rRNA from tobacco ribosomes incubated with PAP [Fig. 2(A) lane 2+]. The RNA was subsequently transferred to a Hybond-N membrane and

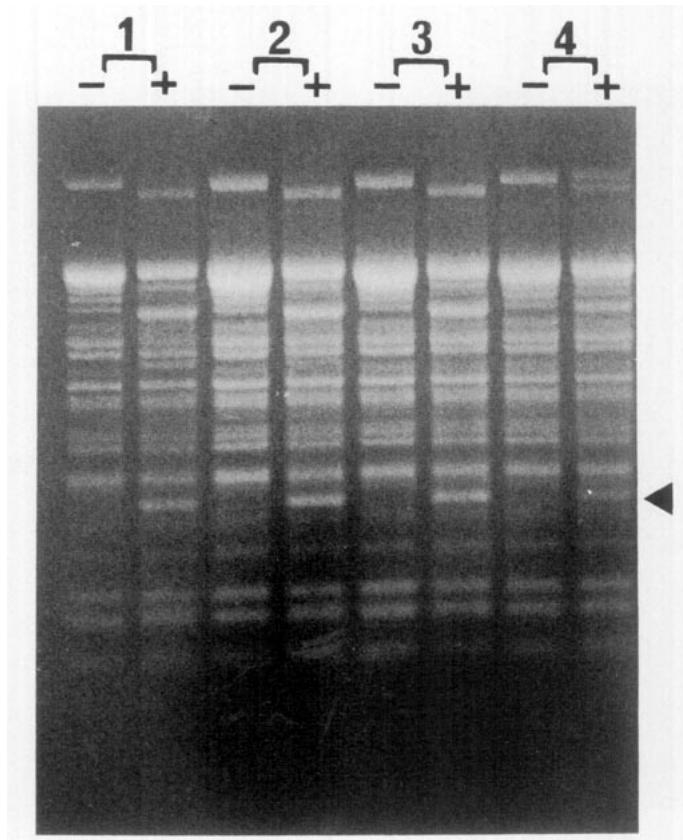


FIG. 1. Effect of PAP on tobacco ribosomes. Tobacco ribosomes were incubated with 100  $\mu$ g PAP (lane 1), 10  $\mu$ g PAP (lane 2), 1  $\mu$ g PAP (lane 3), 0.1  $\mu$ g PAP (lane 4). Following incubation, rRNA was extracted and 3  $\mu$ g samples were treated with aniline and then fractionated on an agarose/formamide gel, the bands were visualized by ethidium bromide staining. + indicates aniline treatment, - indicates no aniline treatment.  $\blacktriangleleft$  indicates the 350-base fragment released from the ribosomes by aniline treatment of the modified rRNA.

hybridized with the PCR-amplified rRNA-specific probe labelled with  $^{32}\text{P}$ . Intact 25S rRNA and the diagnostic fragment of about 350 nucleotides released from rRNA specifically hybridized with the probe [Fig. 2(B) lane 2+]. The diagnostic fragment was much clearer in the Northern blot than in the ethidium bromide-stained gel [Fig. 2(A), (B) lanes 2+].

#### *Time course*

The speed of PAP entering tobacco leaf cells and depurinating 25S rRNA was studied by inoculating PAP onto tobacco leaves and assaying the depurination at different times after inoculation. As shown in Fig. 3, the tobacco 25S rRNA was depurinated by PAP to produce a 350-base RNA fragment after treatment with aniline as early as 5 min after inoculation (Fig. 3 lane 1+) and the depurination remained up to 15 h after inoculation (Fig. 3 lane 6+). RNAs isolated from the leaves inoculated with

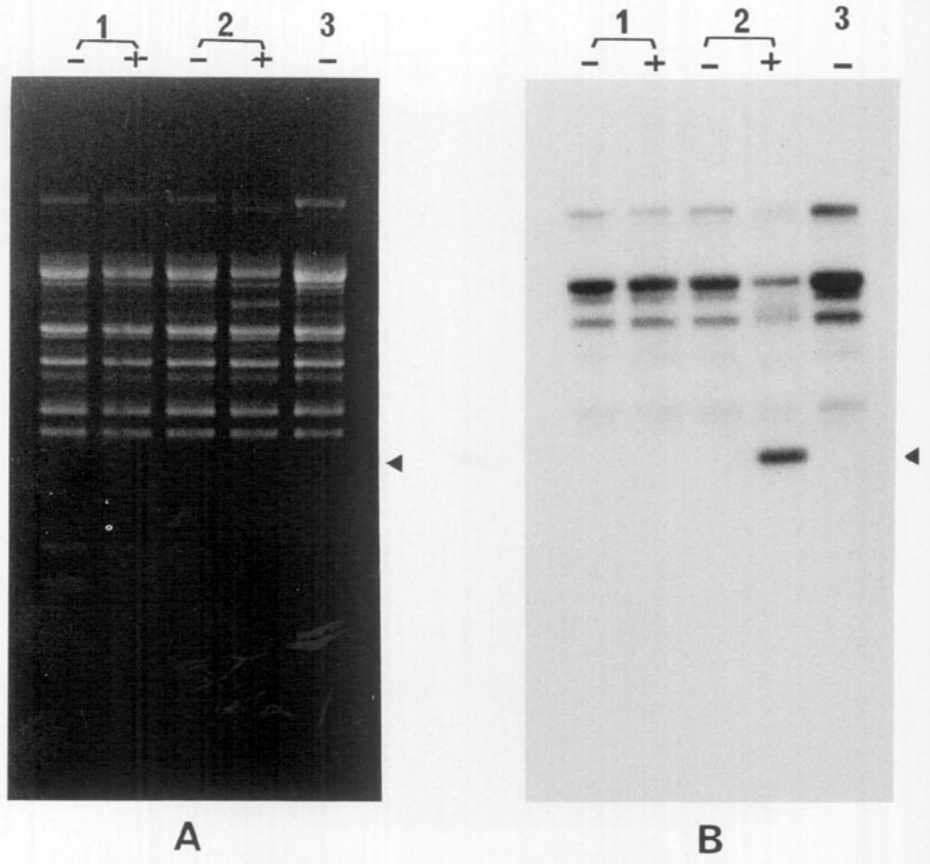


FIG. 2. Specificity of the rRNA-specific probe used to identify the diagnostic fragment released from PAP-modified rRNA. (A) Tobacco ribosomes were incubated without (lane 1) or with  $1 \mu\text{g}$  PAP (lane 2). rRNA was extracted from incubated ribosomes (lanes 1, 2) or untreated ribosomes (lane 3). Two microgram rRNA samples were treated with aniline and fractionated on an agarose/formamide gel. + indicates aniline treatment, - indicates no aniline treatment.  $\blacktriangleleft$  indicates the 350-base fragment released from the ribosomes by aniline treatment of the modified rRNA. (B) The rRNA was Northern blotted onto a Hybond-N membrane and hybridized with the rRNA-specific probe labelled with  $^{32}\text{P}$ . The sample arrangement and markers used were the same as in (A).

TMV alone appear intact (Fig. 3 lane 7+). Infection of tobacco by TMV was completely inhibited by PAP at a concentration of  $1 \mu\text{g ml}^{-1}$ . These results suggest that PAP rapidly enters the cells and inactivates the ribosomes when applied to the leaves and that the depurinated rRNA is not rapidly repaired or recovered.

#### *Dose-response curve*

It was already known [5] that the extent of inhibition of TMV infection by PAP was dependent on the concentration of PAP. In this experiment the extent of depurination of rRNA isolated from tobacco leaves inoculated with PAP at different concentrations was also assayed. As shown in Table 1, 100% inhibition of TMV infection was

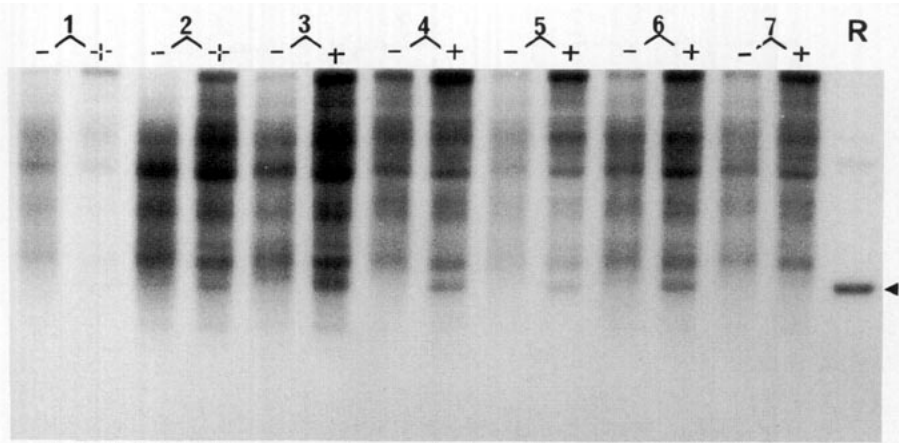


FIG. 3. Effect of PAP on tobacco ribosomes *in vivo* at different times after inoculation. Tobacco leaves were inoculated with a solution containing  $1 \mu\text{g ml}^{-1}$  PAP and  $0.5 \mu\text{g TMV ml}^{-1}$  or  $0.5 \mu\text{g TMV ml}^{-1}$  alone (lane 7). Total RNA was extracted from inoculated leaves collected at 5 min (lane 1), 10 min (lane 2), 1 h (lane 3), 5 h (lanes 4, 7), 10 h (lane 5), and 15 h (lane 6). RNA was fractionated on an agarose/formamide gel after aniline treatment. + indicates aniline treatment, - indicates no aniline treatment. ◀ indicates the fragment released from the ribosomes by aniline treatment of modified rRNA. R indicates tobacco ribosomes incubated with PAP.

TABLE 1  
*Inhibition of TMV infection by different concentrations of PAP*

Conc. PAP* ( $\mu\text{g ml}^{-1}$ )	No. local lesions†	Percentage inhibition
8	0	100
0.8	0	100
0.08	385	71
0.008	758	42
0	1320	

\*PAP at each concentration was mixed with an equal volume of  $1 \mu\text{g TMV ml}^{-1}$  and the mixture was inoculated onto tobacco leaves. TMV ( $0.5 \mu\text{g ml}^{-1}$ ) alone was incubated onto tobacco leaves as a control.

†Number of local lesions on six tobacco leaves.

obtained with  $0.8 \mu\text{g PAP ml}^{-1}$ . The 350-base RNA fragment cleaved from 25S rRNA by aniline treatment was easily visible in Northern blots of RNA isolated from the leaves inoculated with  $8 \mu\text{g PAP ml}^{-1}$  mixed with TMV (Fig. 4, lane 3 +), and a faint band was also seen from extracts of leaves inoculated with  $0.8 \mu\text{g PAP ml}^{-1}$  mixed with TMV (Fig. 4, lane 2 +), but no 350-base RNA fragment was detectable in extracts of leaves inoculated with 80 ng (Fig. 4, lane 1 +) or 8 ng PAP  $\text{ml}^{-1}$  mixed with TMV (data not shown). A 350-base RNA fragment band was also clearly visible from the extracts of leaves inoculated with  $8 \mu\text{g PAP ml}^{-1}$  alone (Fig. 4, lane 4 +). PAP at 80 ng  $\text{ml}^{-1}$  and 8 ng  $\text{ml}^{-1}$  gave 70% and 40% reductions respectively in the numbers of local lesions caused by TMV infection but no 350-bp fragment was detected in 25S rRNA extracted from tobacco leaves inoculated with PAP at the above concentrations. This

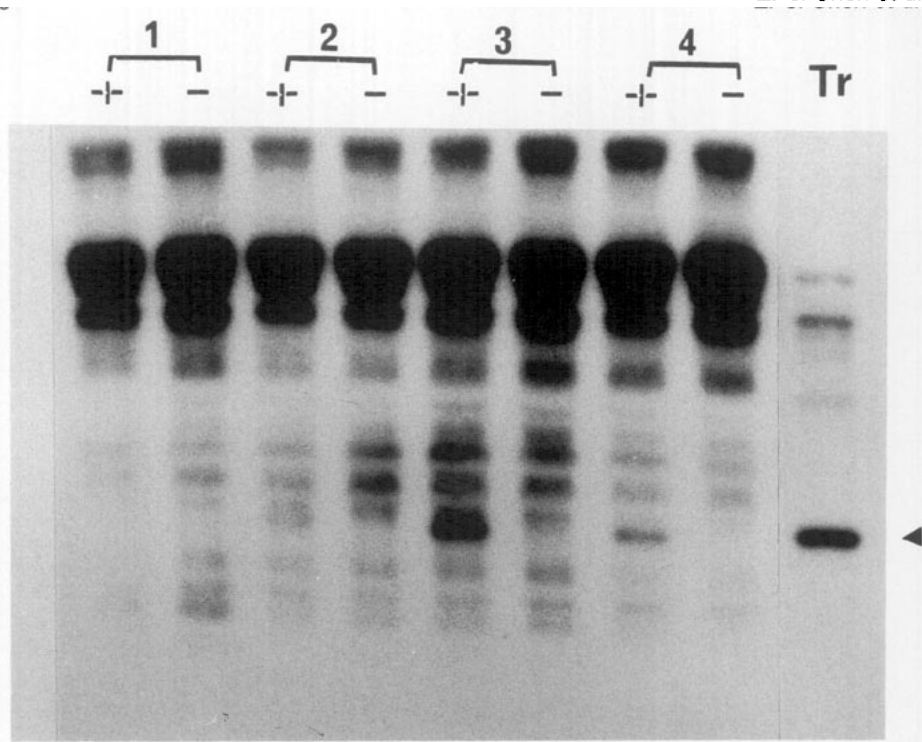


FIG. 4. Effect of PAP at different concentrations on tobacco ribosomes *in vivo*. Tobacco leaves were inoculated with solutions containing PAP at  $80 \text{ ng ml}^{-1}$  (lane 1),  $0.8 \text{ µg ml}^{-1}$  (lane 2),  $8 \text{ µg ml}^{-1}$  (lane 3) and  $0.5 \text{ µg TMV ml}^{-1}$  or  $8 \text{ µg PAP ml}^{-1}$  alone (lane 4). Ribosomes were isolated from inoculated leaves 1 h after inoculation. rRNA was extracted and treated with aniline and then fractionated on an agarose/formamide gel. + indicates aniline treatment, - indicates no aniline treatment. ◀ indicates the fragment released from the ribosomes by aniline treatment of modified rRNA. Tr indicates tobacco ribosomes incubated with PAP.

suggests that the local lesion assay is sensitive enough to show the inhibition of virus infection by PAP but that the proportion of cells that TMV and PAP enter, and thus the amount of depurinated rRNA produced, is so low that it cannot be detected by this assay.

## DISCUSSION

It was found previously that 76% and 60% inhibitions of TMV infection were obtained when  $0.4 \text{ µg PAP ml}^{-1}$  was applied 5 min and 30 min respectively after inoculating tobacco leaves with  $0.5 \text{ µg TMV ml}^{-1}$  and that there was no inhibition when  $0.4 \text{ µg PAP ml}^{-1}$  was applied 50 min after TMV inoculation [5]. This suggests that PAP is effective only at a very early stage of the virus infection process. If inactivation of host ribosomes is the cause of the antiviral activity of PAP, the action of PAP on ribosomes must be before virus replication can begin. The experiments indicate that PAP, once inoculated into tobacco leaves, immediately enters the cells and inactivates the ribosomes. Thus it is likely that the rapid inactivation of ribosomes prevents virus replication at an early stage. The extent of the inhibition of virus



infection and the inactivation of ribosomal RNA were both positively correlated with the concentration of PAP, further supporting the view that when it was inoculated into tobacco leaves, PAP entered the inoculated cells, inactivated the host ribosomes, and thus caused the inhibition of virus replication. Since most viruses need to synthesize replicase using host ribosomes at a very early stage of infection, the infection of all viruses could be inhibited by PAP. This is supported by earlier observations by us and other workers that the infection of all viruses tested, no matter whether they are plant or animal viruses and have DNA or RNA genomes, could be inhibited by PAP.

However, it is still difficult to explain why the pokeweed plant can be infected by viruses if its ribosomes are apparently sensitive to endogenous PAP. Sap from infected pokeweed that will readily infect healthy pokeweed plants by mechanical inoculation will not infect plants of other susceptible species [7]. It is possible that pokeweed ribosomes are susceptible to PAP but less so than ribosomes of other plants, or that there is a factor in pokeweed cells that maintains the ribosomes in such a conformation that they are resistant to PAP attack until the conformation is changed upon isolation from the cell. However, despite all the evidence, PAP's antiviral action does not depend upon the inhibition of host ribosomes.

We thank the Agricultural Genetics Company Ltd (Cambridge, U.K.) for funding the project. We thank Dr M. Hartley for his help with the ribosome depurination assay and Dr D. McCarthy for his helpful discussion and encouragement.

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